CYCLIN D1 as a Genetic Modifier in Hereditary Nonpolyposis Colorectal Cancer

Shashi Bala and Päivi Peltomäki
Division of Human Cancer Genetics, Comprehensive Cancer Center, The Ohio State University, Columbus, Ohio 43210

Abstract

Hereditary nonpolyposis colorectal cancer is associated with inherited defects in DNA mismatch repair. Clinical variation even in cases with identical predisposing mutations suggests the existence of other factors contributing to the phenotype. We addressed the modifying role of the common A/G polymorphism in exon 4 and the alternatively spliced transcripts a and b of the CCND1 gene encoding cyclin D1 in a series of 146 affected carriers of 10 MLH1 and 3 MSH2 mutations. No correlation was observed between a particular allele (A versus G) and age at onset. However, the presence of the variant transcript b in blood/normal mucosa, by multiplex reverse transcription-PCR, was associated with a significantly lower age at onset of colon cancer as compared with individuals with transcript a only (35 versus 46 years; \( P = 0.02 \)). Whereas our data do not support a modifying role of A versus G allele of CCND1, the results do suggest that the relative abundance of a and b transcripts may modify the age at onset of colon cancer in hereditary nonpolyposis colorectal cancer.

Introduction

HNPCC, a familial predisposition to colorectal carcinoma and a variety of extracolonic cancers (1), is associated with germ-line mutations in one of five DNA MMR genes (MLH1, MSH2, MSH6, PMS1, and PMS2). The identification of over 300 predisposing mutations in these genes (2) has facilitated the demonstration of some genotype-phenotype correlations (3–5). However, even kindreds sharing the same genetic predisposition may exhibit significant phenotypic variation, for example, with respect to the age at onset (6), suggesting that other factors besides predisposing mutations must influence the phenotype. Recent studies provide support to the existence of genetic modifiers in HNPCC. Examples of possible modifiers include loci encoding xenobiotic enzymes, NAT1, NAT2, GSTM1, GSTT1 (7–9), or ATM, ataxia-telangiectasia-associated locus (10). Thus, polymorphisms in these loci may modulate the age at onset, penetrance, or location of cancer in MLH1 and MSH2 mutation carriers.

CCND1, located on 11q13 and encoding cyclin D1, is a good candidate for a modifier locus in cancer for several reasons. First, it encodes a protein that plays an important role in cell cycle control in both normal cells and neoplasia (11). Cyclin D1 is the major cyclin involved in transition from G1 to S phase, which is regulated by cyclin-dependent kinases (12, 13). Second, cyclin D1 mRNA exhibits alternate splicing, and translation of the different transcripts (transcripts a and b) results in protein products with nonidentical COOH-terminal domains (14) and with possible functional differences (15). Transcript a is normally spliced, whereas transcript b is a variant transcript reading into intron 4 and skipping exon 5. Third, a G to A polymorphism at the splice donor site of exon 4 has been postulated to increase alternate splicing (transcript b; Ref. 14). These observations together lay a theoretical background for a possible role of CCND1 as a cancer modifier gene. Indeed, Betticher et al. (14) reported that patients with resected non-small cell lung cancer and AG or AA genotype showed a shorter relapse-free survival as compared with those with the GG genotype. Most interestingly from the present point of view, Kong et al. (16) recently reported an earlier age of onset by 11 years in HNPCC patients who were homozygous or heterozygous for the mutant allele (A) as compared with patients who were homozygous for the normal allele (G).

The present report addresses the significance of cyclin D1 A/G polymorphism and mRNA expression as modifiers of clinical characteristics of HNPCC. The study was based on a large series (\( n = 146 \)) of affected carriers from clinically well-characterized families segregating mutations in the DNA MMR genes MSH2 or (mostly) MLH1. Mutation-negative individuals (\( n = 186 \)) from the same families served as controls.

Materials and Methods

Patients and Samples. A total of 146 patients from 53 Finnish HNPCC families who had a predisposing mutation in MLH1 (10 mutations; 141 patients) or MSH2 (3 mutations; 5 patients) were included. The MLH1 mutations were as follows: (a) mutation 1, 3.5-kb genomic deletion affecting codons 578–632 of exon 16 and flanking intron sequences; (b) mutation 2, g > a at 454-1 at splice acceptor of exon 6; (c) mutation 3, genomic deletion of exons 3–5; (d) mutation 4, G > C at 1975 (codon 659) of exon 17; (e) mutation 5, g > c at 1409 + 1 at splice donor of exon 12; (f) mutation 6, T > G at 320 (codon 107) of exon 4; (g) mutation 9, g > a at 1039-1 at splice acceptor of exon 12; (h) mutation 10, g > a at 1559-1 at splice acceptor of exon 14; (i) mutation 11, C > T at 1975 (codon 659) in exon 17; and (j) mutation 12, a > c at 1559-2 at splice acceptor of exon 14. The MSH2 mutations were as follows: (a) mutation 6, ins TG at 1860 (codon 619) of exon 12; (b) mutation 8, del CA at 1550 (codon 518) of exon 10; and (c) mutation 13, g > a at 1387-1 at splice acceptor of exon 9. The present series is enriched with MLH1 mutation 1 (97 patients) and is therefore shown separately, when appropriate, whereas other mutations were pooled. Mutation-negative individuals (\( n = 186 \)) from the same families were used as controls. DNA and RNA were extracted from EBV immortalized cell lines derived from the patients’ blood and, in some cases, from the normal colonic mucosa and fresh frozen tumor tissue. All human samples were obtained after informed consent according to the institutional guidelines.

Detection of A/G Polymorphism. The A/G polymorphism in codon 242 of exon 4 of CCND1 was ascertained by single-strand conformational polymorphism analysis using primers 1 and 2 reported previously (16). The genotype of the three polymorphic types (AA, AG, and GG) was further confirmed by sequencing.

Allele-specific Expression Analysis. This analysis was carried out on the available 31 RNA samples from lymphoblastoid cells and/or normal mucosa from patients with colon cancer and from 12 RNAs of colon tumor tissues. Total RNA (2.5 μg) was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Promega), followed by two rounds of RT-PCR as reported previously (14). The final PCR products were digested with SacF1 and analyzed by agarose gel electrophoresis.

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2 To whom requests for reprints should be addressed, at the Division of Human Cancer Genetics, Comprehensive Cancer Center, Ohio State University, 690 Medical Research Facility, 420 West 12th Avenue, Columbus, OH 43210. Phone: (614) 688-4493; Fax: (614) 688-4245; E-mail: peltomaki-1@medctr.osu.edu.
3 The abbreviations used are: HNPCC, hereditary nonpolyposis colorectal cancer; RT-PCR, reverse transcription-PCR; LOH, loss of heterozygosity; MMR, mismatch repair.
4 www.nfdht.nl.
restriction enzyme and visualized on 3.5% NuSieve agarose gel (BioWhittaker Molecular Applications), and the results were scored for allelic imbalance.

**Multiplex RT-PCR.** To study the relative expression of transcripts a and b simultaneously, multiplex RT-PCR was performed using primer sets Cy10/Cy6 (for transcript a) in combination with Cy10/Cy32 (for transcript b; Ref. 14). We used 55°C annealing temperature for 35 cycles, followed by a second-round PCR using 1:50 dilution and primer sets Cy1F/Cy4R (for transcript a) and Cy1F/Cy27 (for transcript b) at 60°C annealing temperature with 32 cycles. Cy27 was as reported previously (14), whereas the other primers were designed by us [Cy1F, 5'-GGAGCTGCTCCTGGTGAACAA-3', Cy4R, 5'-CTCTGGGGTGCTCATGTTGC-3' (nucleotides 936–955 in exon 5)]. The PCR products were resolved on a 3% NuSieve agarose gel and subject to overnight blotting on Zeta-Probe (Bio-rad). Nested primer Cy1F was end-labeled with [γ-32P]-dATP (Pharmacia) and used as a hybridization probe at 60°C. The blot was processed and exposed to Kodak X-OMAT film for 45 min. Autoradiographic signals corresponding to 389 and 324 bp indicated the expression of transcripts a and b, respectively.

**LOH Analysis.** Tumor DNA was studied in comparison with the matched normal mucosa by amplifying the polymorphic exon 4 region with primers Cy26/Cy27 (14), followed by F1 (New England Biolabs) restriction digestion.

**Statistical Analysis.** The comparison between the observed and expected allele frequencies in the mutation-positive and -negative groups was studied by $\chi^2$ test. The difference between the mean ages at onset in patients with transcripts a versus b was evaluated using two-tailed t test.

**Results**

**Cyclin D1 Genotype Frequencies.** The genotype frequencies were studied in 146 MLH1/MSH2 gene mutation-positive HNPCC patients and 186 mutation-negative individuals from the same families (Table 1). In the mutation-positive group, the frequency of A and G alleles was 0.42 and 0.58, respectively, which is consistent with the known sequence differences in the exon 4 region. Among others, these results verified that the EBV transformation of lymphoblastoid cells did not alter their splicing pattern compared with the nontransformed cell types.

**Correlation of Genotypes with Clinical Features.** No correlation was observed between a particular allele (A vs. G) and age at onset of cancer (Table 2). However, the average age at onset of cancer was higher in the heterogeneous individuals (AG, 46 years) as compared with individuals homozygous for the normal allele (GG, 41 years) or the variant allele (AA, 39 years). Similarly, the average age at onset of HNPCC-related extracolonic cancer was higher in heterozygotes as compared with the homozygotes (Table 2). The same trends were observed when the analysis was limited to only carriers of mutation 1, the largest individual group, to reduce heterogeneity associated with different predisposing mutations. There was no correlation between the different genotypes and colonic location of cancer or occurrence of extracolonic cancer.

**Allelic Origin of Alternately Spliced Transcripts.** In this analysis, the known sequence differences in the exon 4–5 region were used to design reactions specific to each of the transcripts, which were amplified individually, and the allelic origin (A versus G) of the transcripts was then determined by RFLP analysis as described in “Materials and Methods.” Both transcripts (a and b) were invariably amplified from all samples (data not shown). In accordance with a previous report (14), allele G spliced to transcripts a and b, whereas allele A spliced mostly into transcript b (comprising intron 4 sequence). Among others, these results verified that the EBV transformation of lymphoblastoid cells did not alter their splicing pattern compared with the nontransformed cell types.

**Multiplex RT-PCR Analysis of Transcripts a and b and Correlation with Genotypes.** The allele-specific analysis described above was suitable for efficient individual amplification of each transcript, but not for their relative quantification. We therefore designed multiplex RT-PCR analysis using a common forward primer (from exon 4) and different reverse primers (corresponding to the exon 5 cDNA sequence for transcript a and intron 4 DNA sequence for transcript b). The primers and reactions were carefully designed and optimized not to interfere with each other (see Fig. 1), thereby allowing the simultaneous analysis of the two transcripts. As shown in Table 3, the normally spliced transcript a was predominant over the variant transcript b in blood/normal mucosa from HNPCC patients with colon cancer, irrespective of the CCND1 genotype. Transcript a alone was visible in 65% of the cases, and transcript b was visible in 10% of the cases, whereas coexpression (a + b) was seen in 26% of the cases.

**Comparison of Normal and Tumor Tissue.** Paired samples of normal and tumor tissue DNA were available from 14 cases (11 colorectal cancers, 2 ovarian cancers, and 1 endometrial cancer) for LOH analysis; no LOH at CCND1 was observed in 7 informative cases (data not shown). Multiplex RT-PCR analysis for the expression of transcripts a and b was carried out on 12 colorectal cancers (Table 3), including 7 cases with paired normal and tumor RNA samples.
available (Fig. 1). The results showed either expression of transcript a only or coexpression of transcripts a + b in tumor tissue RNA. In two cases (cases 4 and 7 in Fig. 1), a clear shift in the relative abundance of transcripts a and b was observed in normal versus tumor tissue. Because the tumors showed no LOH (case 7 was not informative), the expression imbalances were likely to have arisen at the transcriptional level.

**Correlation of Splice Variants with Age at Onset.** The expression of the two transcripts a and b in blood/normal mucosa was analyzed with respect to the age at onset of colon cancer (Table 4). When transcript a was exclusively expressed, the average age at onset was 46 years (range, 29–69 years), whereas it was as low as 24 years (range, 19–30 years) when only transcript b was expressed (by t statistics, \(P = 0.003; df = 21\)). In general, the presence of transcript b, alone or in combination with transcript a, showed a significant association with early average age at onset compared with patients with transcript a only (35 versus 46 years, \(P = 0.02; df = 29\)).

**Discussion**

Despite the abundance of reports on MMR gene mutations in HNPCC, there is no precise explanation for variable phenotype of patients sharing the same predisposing mutation. For this reason, we attempted to study the possible modifying role of **CCND1** in Finnish HNPCC kindreds, in which a vast majority of individuals had been identified with an ancestral founding mutation in **MLH1** (mutation 1) and with variable clinical features (6). We could not confirm the observation by Kong et al. (16) of a dominant allele A conferring an increased age-associated colon cancer risk on individuals with AA and AG genotypes of **CCND1**. Instead, in our study, the age at onset of colon cancer (and extracolonic cancer) was lower by 5–6 years in both types of homozygotes (AA and GG) as compared with heterozygotes (AG).

There are several possible reasons for the discordance between the findings of the present study and that of Kong et al. (16). First, the predisposing mutations may mask the modifying effects of **CCND1** by being strong independent determinants of clinical outcome or, alternatively, because the MMR genes, also participate in cell cycle control (17), by possibly interacting with **CCND1**. Thus, it may be important that the study of Kong et al. (16) was based predominantly on **MSH2** mutation carriers (57 of 86 patients, 66%), whereas our investigation was based on **MLH1** mutation carriers (141 of 146 patients, 97%). Furthermore, a significant fraction of patients studied by Kong et al. (16) had missense mutations (40 of 86 patients, 47%) as compared to only 8 of 146 (5%) in our study; the remaining mutations were truncating mutations. The pathogenicity of missense mutations is sometimes questionable, whereas truncating mutations are likely to be pathogenic. Second, our patient series was larger (146 affected mutation carriers versus 49 affected + 37 unaffected carriers), and, unlike the affected carriers studied by Kong et al. (16), our population was in Hardy-Weinberg equilibrium and therefore less likely to be biased. Third, there may be an ethnic or environmental variation that may confound the results. Importantly, in our investigation, carriers of mutation 1 formed as homogeneous a population as possible, having common ancestry and shared geographic origin (18), and the results were the same as in the pooled series, thus emphasizing the reliability of our data.

In our investigation, the presence of the variant transcript b in blood/normal mucosa was associated with a significantly lower age at onset of colon cancer as compared with individuals with the normally spliced transcript a only. Transcript b that lacks the PEST destruction box may have a longer half-life than transcript a (14) and may thereby promote the proliferative effects of **CCND1**. Importantly, our multiplex RT-PCR analysis showed that transcript a was predominant over b in all three genotypes (AA, AG, and GG) and especially in individuals with the AG genotype (Table 4), thus being compatible with our finding of a later age of onset in the latter group. In accordance with our results, a recent study on 100 sporadic colon cancers failed to demonstrate any association between **CCND1** genotype and overall

**Table 3 Expression of splice forms a and b in relation to CCND1 genotypes in MMR gene mutation carriers diagnosed with colon cancer (normal and tumor results are treated here as independent data sets).**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Blood or normal mucosa (n = 31)</th>
<th>Tumor (n = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>a (60%)</td>
<td>b (40%)</td>
</tr>
<tr>
<td>AA</td>
<td>3 (60%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>AG</td>
<td>11 (69%)</td>
<td>1 (6%)</td>
</tr>
<tr>
<td>GG</td>
<td>6 (60%)</td>
<td>2 (20%)</td>
</tr>
<tr>
<td>Total</td>
<td>20 (65%)</td>
<td>3 (10%)</td>
</tr>
</tbody>
</table>

**Table 4 Correlation of cyclin D1 splice variants with age at onset.**

<table>
<thead>
<tr>
<th>Transcripts expressed in blood/normal mucosa</th>
<th>Age at onset of colon cancer (yrs)</th>
<th>Average ± SD</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Either a or b expressed</td>
<td>46 ± 11</td>
<td>29–69</td>
<td></td>
</tr>
<tr>
<td>a only (n = 20)</td>
<td>46 ± 11</td>
<td>29–69</td>
<td></td>
</tr>
<tr>
<td>b only (n = 3)</td>
<td>24 ± 5</td>
<td>19–30</td>
<td></td>
</tr>
<tr>
<td>Both a and b expressed</td>
<td>35 ± 7</td>
<td>27–45</td>
<td></td>
</tr>
<tr>
<td>a predominant over b (n = 5)</td>
<td>35 ± 7</td>
<td>27–45</td>
<td></td>
</tr>
<tr>
<td>b predominant over a (n = 2)</td>
<td>55 ± 9</td>
<td>46–64</td>
<td></td>
</tr>
<tr>
<td>a and b equally expressed (n = 1)</td>
<td>30</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
survival; additionally, CCND1 genotype was unrelated to the level of expression of cyclin D1 protein, which unlike genotype, was a prognostic indicator (19).

In summary, whereas the data available to date suggest that cyclin D1, with its polymorphic nature and the alternatively spliced forms, may contribute to the clinical characteristics of HNPPC and other cancers, the relationships are complex, and more studies are needed to explore the biological basis of the proposed interactions.

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References

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